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Are Highways Stressful for Pikas?: Analysis of Stress Hormones of the American Pika (*Ochotona princeps*) Living Adjacent to Interstate 90 in the Cascade Range of Washington State

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ARE HIGHWAYS STRESSFUL FOR PIKAS?: ANALYSIS OF STRESS HORMONES OF THE AMERICAN PIKA (*Ochotona princeps*) LIVING ADJACENT TO INTERSTATE 90 IN THE CASCADE RANGE OF WASHINGTON STATE

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by

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ABSTRACT

ARE HIGHWAYS STRESSFUL FOR PIKAS?: ANALYSIS OF STRESS HORMONES OF THE AMERICAN PIKA (*Ochotona princeps*) LIVING ADJACENT TO INTERSTATE 90 IN THE CASCADE RANGE OF WASHINGTON STATE

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Roads often disrupt ecosystem connectivity, harming many wildlife populations. Wildlife crossing structures perforate the barrier-effect of roads, providing safe passage for species dispersing across roads, thereby reconnecting ecosystems. Some wildlife species live in modified habitats along roads and in habitat features within wildlife crossing structures, but their fitness in these potentially stressful environments is poorly understood. Chronic stress alters behavior, reduces reproduction, and has been linked to reduced survival. Animals affected by chronic stress in road-adjacent habitats could reduce the ability of wildlife crossing structures to improve connectivity across roads. I assessed stress hormone concentrations in a low-mobility species, the American Pika (*Ochotona princeps*), living in anthropogenic rock embankment along Interstate 90 (highway) in the Cascade Range of Washington. I extracted fecal glucocorticoid metabolite (fGCM) concentrations from fresh fecal samples to determine chronic stress

levels in pikas living adjacent to the highway compared with those living in similar rock embankment in a rails-to-trails state park, and in natural rocky sites. I evaluated potential stressors in the highway and rails-to-trail habitats by measuring microclimate proxies (environmental temperatures, elevation, and longitude) and noise levels. A linear mixed-effects model was used to test for differences in fGCM concentrations among habitats and assess the potential effects of these environmental variables on stress. Pikas in the highway habitat had the lowest fGCM levels among the 3 habitats. Stress hormone concentrations showed significant relationships with longitude and noise, but not with elevation or mean daily temperatures. I hypothesize that the lowered fGCM levels of highway pikas may be a result of downregulation of the stress response under chronic exposure to stressors, potentially including highway noise. My results offer insights into the physiological response of pikas to potentially stressful environments disturbed by humans and may provide a good indicator of stress levels expected for pikas colonizing wildlife crossing structures now being constructed to improve wildlife connectivity across I90.

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CHAPTER I

LITERATURE REVIEW

Habitat Fragmentation

High-quality habitat is essential for biodiversity and healthy ecosystems (Loreau and others 2001; Hooper and others 2005; Roques and others 2016), and as humans degrade habitats, species are harmed from habitat loss and fragmentation (Reed 2004). Habitat loss reduces the usable space for species. For example, deforestation and conversion of grassland to agricultural land eliminate large continuous sections of habitat, causing a loss of biodiversity (Pykälä 2019). Fragmentation, the separation and isolation of 1 continuous habitat into multiple smaller habitats, may not cause the direct loss of substantial habitat area, but the indirect consequences of a fragmented landscape damage biodiversity and ecosystems (Krauss and others 2010).

The effects of habitat fragmentation on ecosystems depend on the degree of isolation, the edge habitat created, the size of the fragment, and the duration of fragmentation (Krauss and others 2010; Ibáñez and others 2014). Researchers often reach conflicting conclusions on the effects of habitat fragmentation (Fahrig 1997). The choice of target species can mislead researchers about the damage caused to an entire ecosystem. For example, fragmentation creates more edge habitat; therefore, studies that focus on edge habitat specialists are likely to find a benefit to those species (Barrera and others 2015). A broader scope often shows that fragmentation negatively affects nutrient retention, species richness, community composition, and biodiversity

(Haddad and others 2015). Greater fragmentation as a result of more impermeable barriers subdivides populations, increases genetic isolation, and elevates the risk of inbreeding, potentially leading to extinction (Andersen and others 2004; Reed 2004).

Metapopulations and Connectivity

By isolating populations, habitat fragmentation can also create metapopulations, or populations of populations (Hanski and Gilpin 1991; O'Grady and others 2006). Through the lens of metapopulation ecology, we can view fragmented habitats as subpopulations as long as some connectivity remains (Hanski 1998). Connectivity allows for dispersal and migration, providing opportunities to transfer genes among subpopulations (Hanski and others 1995). Some habitat patches are less successful than others at sustaining a sub-population, while other habitats produce more individuals than can be supported by the habitat. Individuals in the latter habitats are forced to disperse from their natal patch to neighboring patches, introducing genetic variability (Hanski 1998). Connectivity allows for patchy areas of suitable habitat to provide populations with access to resources, mates, and genetic diversity that are not otherwise available (Pratt and others 2018). Conversely, fragmented populations of inadequate size with limited connectivity risk genetic stagnation (Macías-Duarte and others 2019). Small populations are subject to genetic drift (Nei and Tajima 1981), and isolated populations inbreed, reducing the ability to adapt to a changing environment (O'Grady and others 2006). Inbreeding can lead to the accumulation of deleterious

alleles and limit a population's resistance to disease (Lynch and Walsh 1998). Species also become less resilient to the inevitable pressures caused by climate change and natural disasters (Lande and Shannon 1996). This can reduce survival rates and reproductive success, leading to an extinction vortex (Blomqvist and others 2010). Habitat fragmentation decreases the connectivity necessary to maintain metapopulations across a landscape. In summary, connectivity injects much-needed genetic diversity into populations to save species from negative population trends, local extirpation, or even extinction (Keyghobadi 2007).

Fragmentation by Roads

Mechanisms responsible for habitat fragmentation include deforestation, urbanization, and our ubiquitous system of roads (Fahrig 1997; Ibáñez and others 2014; Haddad and others 2015). Roads are partially permeable barriers, but they restrict movement enough to fragment populations and habitats (Shepard and others 2008). The size of a road and, more importantly, the traffic volume contribute to the intensity of the barrier effect (Jaeger and others 2005; Marsh and Jaeger 2015). Roads are barriers to animal movement in 2 ways: through behavioral avoidance and direct mortality from vehicle collisions (Forman and Deblinger 2000). Animals avoid well-traveled roads due to the noise, light, and the often-abrupt change in habitat (Forman and Alexander 1998). The area surrounding a road has direct effects on animal behavior, but the indirect effects extend well into neighboring habitats.

The road-effect zone is the area away from a road edge that is ecologically affected by the road (Forman and others 1997; Shanley and Pyare 2011). These zones alter local hydrology, erosion, nutrient cycling, species composition, and dispersal (Forman and Alexander 1998). The size of the road-effect zone depends on the type of road, amount of traffic, topography of the landscape, and species under consideration (Forman and Deblinger 2000), but is generally estimated to be from 250 to 1000 m wide (Eigenbrod and others 2009; Shanley and Pyare 2011). Invasive plant species are spread along roadsides, drastically altering the species composition in these areas (Angold 1996; Kowarik and von der Lippe 2011). These plants then spread into the adjacent communities (Gelbard and Belnap 2003). Habitats fragmented by roads have an increased proportion of edge habitat (Forman and others 2003). This reduces the amount of habitat available to forest interior species that require certain minimum expanses of continuous habitat (Forman and others 2003), and can serve as a barrier to forest interior species unwilling to transition between habitat types (Alharbi and Petrovskii 2016). An estimated 20% of the world's remaining forests are within 100 m of an edge, and 70% are within 1 km of an edge (Haddad and others 2015).

Direct mortality poses a lower ecological threat than behavioral avoidance but is still an important issue in road ecology (Forman and Alexander 1998). Vehicle strikes on amphibians can lower population density in the surrounding area (Fahrig and others 1995). In the Lake Tahoe Basin of Sierra Nevada, vehicle collisions with black bears increase the mortality rate above the birth rate, contributing to the creation of a

population sink (Beckmann and Lackey 2008). Sensitive species are even more severely threatened by vehicle collisions. Direct mortality is the single biggest threat to the survival of 21 federally threatened or endangered species, including the San Joaquin Kit Fox (*Vulpes macrotis*), Florida Panther (*Felis concolor coryi*), Desert Tortoise (G*opherus agassizii*), American Crocodile (*Crocodylus acutus*), California Tiger Salamander (*Ambystoma californiense*), Hawaiian Goose (*Branta sandvicensis*), and Florida Scrub Jay (*Aphelocoma coerulescens*) (Huijser and others 2008).

Conversely, effects of roads may also be minimal or even beneficial (Huijser and Clevenger 2006; Beckmann and Hilty 2010; Bhardwaj and others 2019). In Yellowstone National Park, Moose (*Alces alces*) have begun to avoid their main predators, Brown Bears (*Ursus arctos horribilis*), by giving birth near roads (Berger 2007). Roadkill also provides foraging opportunity to scavengers. For example, populations of Common Ravens (*Corvus corax*) increased with road density (Knight and others 1995). To responsibly evaluate road fragmentation as beneficial or detrimental, we should evaluate their effect on multiple species. When considering the impacts to entire ecosystems, the majority of studies find that roads harm connectivity and therefore harm ecological communities (Keyghobadi 2007; Haddad and others 2015). To mitigate the harmful effects of roads on ecosystems, we must reconnect habitats, restoring the ability of wildlife to disperse across roads without impediment.

Wildlife Crossing Structures

Animals are driven to disperse across a landscape for foraging and mating opportunities. Roads deter many animals from dispersing across them but for some animals, the urge to move to new habits overpowers the fear of a road. Inevitably, some areas of roads are more prone to animal-vehicle collisions. This is dangerous and often fatal for both animals and humans (Huijser and others 2008). Ecologists and transportation departments employ many mitigation efforts, such as hazing, reflectors, animal detection systems, and warning signs to reduce these collisions (Huijser and others 2008). These techniques are used throughout the United States with mixed results (Forman and others 2003). The most effective way to improve motorist safety is to install wildlife crossing structures (WCS) and fencing that funnels animals towards them (Forman and others 2003; Sullivan and Messmer 2003). WCSs decrease wildlife-vehicle collisions and perforate the road barrier. By building wildlife overpasses and underpasses, we can allow wildlife to safely move across highways with many lanes and high traffic volumes (Clevenger and Huijser 2011).

Species are drawn to use a WCS based on its design and placement (Clevenger and Waltho 2000). Overcrossings are open-air structures that span above a highway, while undercrossings allow animals to pass underneath a roadway and can take the form of small culverts (< 0.5 m diameter), large culverts (>3 m diameter), or box culverts with natural or artificial bottoms (Ruediger 2001; McDonald and St. Clair 2004). Undercrossings can be wide enough where they require a large bridge (> 100 m long) for

the roadway over them. Different species prefer different WCS types, lengths, heights, and widths (Rytwinski and others 2015). Many large carnivores and ungulates often prefer short crossings under tall and wide structures, however Mountain Lions (*Puma concolor*) and Black Bears (*Ursus americanus*) are partial to more enclosed undercrossings (Clevenger and Waltho 2005). Small mammals readily use pipe culverts and other small structures (Martinig and Bélanger-Smith 2016). The most effective structures do not target single species, but rather provide suitable passage for the most species possible (Clevenger and Waltho 2000; Beier and others 2008). For example, extended stream crossings are passages over a stream or creek that include space on either side for a wide variety of terrestrial animals (Ruediger 2001; Niemi and others 2014).

Connectivity is improved by the appropriate type of WCS, but to adequately mitigate the barrier effect of roads we need to place WCS strategically in a landscape (Clevenger and Ford 2010). This is done by identifying wildlife hotspots along a road (Gomes and others 2009). Road ecologists use 2 main approaches to locate hotspots: spatial modeling using landscape features, and road surveys to find high frequencies of roadkill (Boyle and others 2017). Landscape-based hotspot identification uses spatial models to identify areas or features that are highly likely to encourage animal movement (Larkin and others 2004; Koen and others 2014; Santini and others 2016). Ecologists target streams, valleys, and known migration corridors to help locate sites for WCSs where they will perform best (Huijser and others 2008). Often, these hotspots can

be found at ecotones (Gunson and others 2011; Barthelmess 2014), or in areas where similar habitat exists on either side of the road (Langen and others 2012). Hotspots can also occur in areas of high traffic volumes, or when characteristics of the road (width, visibility, and terrain) make crossing attempts less successful (Gunson and others 2011; Langen and others 2012; Barthelmess 2014).

Identifying hotspots using roadkill requires field surveys to document wildlife-vehicle collisions (Cureton and Deaton 2012). Roadkill hotspots are often species-specific, and their locations and frequencies fluctuate seasonally (Crawford and others 2014). Road ecologists can identify areas common to many species by surveying throughout the year and using historical data (Barthelmess 2014). Road surveys should be conducted frequently to accurately identify the number of wildlife-vehicle collisions. Scavengers remove small carcasses within 1 day and can remove a large carcass from the road within a week (Santos and others 2011). Additionally, wildlife-vehicle collisions are capable of depressing populations, eventually resulting in lower counts during roadkill surveys (Teixeira and others 2017). This could cause ecologists to ignore a population or community that has few roadkills but might highly benefit from a WCS (Eberhardt and others 2013).

Arguably, the best approach for WCS placement uses both techniques to complement each other. Landscape-based approaches successfully identify regions, and road surveys pinpoint specific sites for WCSs (Boyle and others 2017). The most effective WCSs incorporate the expertise of ecologists and engineers across a spectrum

of disciplines. Once ecologists and departments of transportation use landscape spatial models and roadkill surveys to determine the ideal location for structures, they can collaborate to implement the most effective WCS design for the targeted species, or groups of species. By incorporating these recommendations into road mitigation efforts, agencies can simultaneously improve motorist safety and enhance ecological connectivity. One project using these techniques is currently underway along Interstate 90 in the Cascade Range of central Washington State.

Interstate 90 Snoqualmie Pass East Project

Interstate 90 (I90) is the longest interstate in the United States, spanning 4,964 km from Boston, MA to Seattle, WA (FHA 2017). While traversing Washington State, I90 winds through Snoqualmie Pass in the Cascade Range (the Cascades). On average, 31,000 vehicles per day use this mountain pass (WSDOT 2019). I90 is an important transportation corridor that connects the eastern and western portions of Washington. In doing so, the interstate bisects the Okanogan-Wenatchee National Forest. This region is vital to the east-west cultural and economic connectivity of Washington, but also to the north-south ecological connectivity of species and ecosystems through the Cascades (WSDOT 2006). The Washington Department of Transportation (WSDOT) has identified a 24-km stretch of I90 between Hyak and Easton as high priority for improving transportation flow, motorist safety, and ecological connectivity (WSDOT 2006).

Construction along I90 began in 2008 and is ongoing (as of 2019) in an effort to reduce the risk from avalanches and rock falls, update failing infrastructure, and improve overall safety for drivers (WSDOT 2006). WSDOT is widening the highway from 4 to 6 lanes to reduce congestion. Simultaneously, WSDOT is improving connectivity to support biodiversity in the project area and reduce the risk of wildlife-vehicle collisions (Clevenger and others 2008). The I90 Snoqualmie Pass East (SPE) project originated from an impressive level of collaboration among nearly 2 dozen organizations, institutions, and state, federal, and tribal agencies. Groups dedicated to the success of the I90 SPE project include WSDOT, United States Forest Service, Washington Department of Fish and Wildlife, United States Fish and Wildlife Service, Conservation Northwest, Central Washington University, and I90 Wildlife Bridges Coalition, to name a few.

The ecological goal of the I90 SPE project is to install a variety of WCSs to improve connectivity for targeted functional species groups (Long and others 2012). To provide desirable crossing opportunities to myriad species, WCSs of different dimensions and sizes are being installed (Clevenger and Huijser 2011). Small round pipes and box culverts (< 1.5 m wide) intended for drainage appeal to amphibians, reptiles and some small mammals (McDonald and St. Clair 2004; Martinig and Bélanger-Smith 2016). Medium-sized extended stream crossings (2-30 m wide) allow for passage of fish as well as larger, highly mobile mammals such as deer and coyotes (Huijser and others 2008). The largest structures being built are extended stream undercrossings. They are 30- to 300-m long bridges built over creeks in areas of heavy animal activity (Mark

Norman, WSDOT, pers comm.). Large overpasses are also being built, spanning across the highway, offering a natural, unobstructed environment open to the same elements as the connecting forest. These larger structures appeal to the most diverse array of species (Ruediger 2001; Lesbarrères and Fahrig 2012; Niemi and others 2014).

Within these structures and connecting to adjacent habitats, the I90 SPE project involves installing habitat features typical of the surrounding landscape. Participants are planting vegetation; adding rock piles, brush piles, and fallen logs; and when possible, including aquatic features such as streams. The SPE project aims to encourage medium-sized and large animals to use the WCSs as dispersal corridors, while also enhancing connectivity for small animals with low mobility by providing habitat that allows them to colonize the structures. By including a focus on low-mobility species, the SPE project is aiming to provide more complete connectivity across all taxa and trophic levels (Clevenger and Waltho 2000; D'Amico and others 2015; Santini and others 2016).

Habitats near (and within) WCS are inherently close to roadways and within the road-effect zone where fitness can be reduced. These habitats can be stressful for low-mobility species because most of their home range can be highly affected by stressors from the road (Navarro-Castilla and others 2014). By understanding how habitats along roads contribute to stress, we can better evaluate the fitness of species expected to live near or within WCSs.

Wildlife Stress Response

All vertebrates experience some form of stress (Breuner and others 2013), defined as the physiological response to a threat (Boonstra and others 1998; Sapolsky 2002; Reeder and Kramer 2005). Threats, or stressors, can be environmental, physical, or psychological (Wasser and others 1997; Boonstra and others 1998). Examples of environmental stressors include exposure to extreme heat or cold, while physical stressors include a direct assault from a predator, or disease. A psychological stressor is the fear of a predator, whether that fear be real or only perceived. The presence of a stressor activates the stress response (Sheriff and others 2011), a collection of cascading physiological reactions meant to aid in survival and then eventually bring the body back to a state of homeostasis (Romero 2002). It originates with the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1), resulting in the release of a category of stress hormones known as glucocorticoids (GCs) (Munck and others 1984; Sapolsky 2002; Tsigos and Chrousos 2002). These hormones are expressed as either corticosterone or cortisol, depending on the species (Sapolsky 2002; Sheriff and others 2009). Cortisol is found in humans (Romero 2004), Snowshoe Hares (*Lepus americanus*) (Boonstra and others 1998), Koalas (*Phascolarctos cinereus*) (Davies and others 2013), and most primates and carnivores (Touma and Palme 2005). Most lagomorphs (Wilkening and others 2013), rodents (Erickson and others 2017), birds, and reptiles (Touma and Palme 2005) release corticosterone.

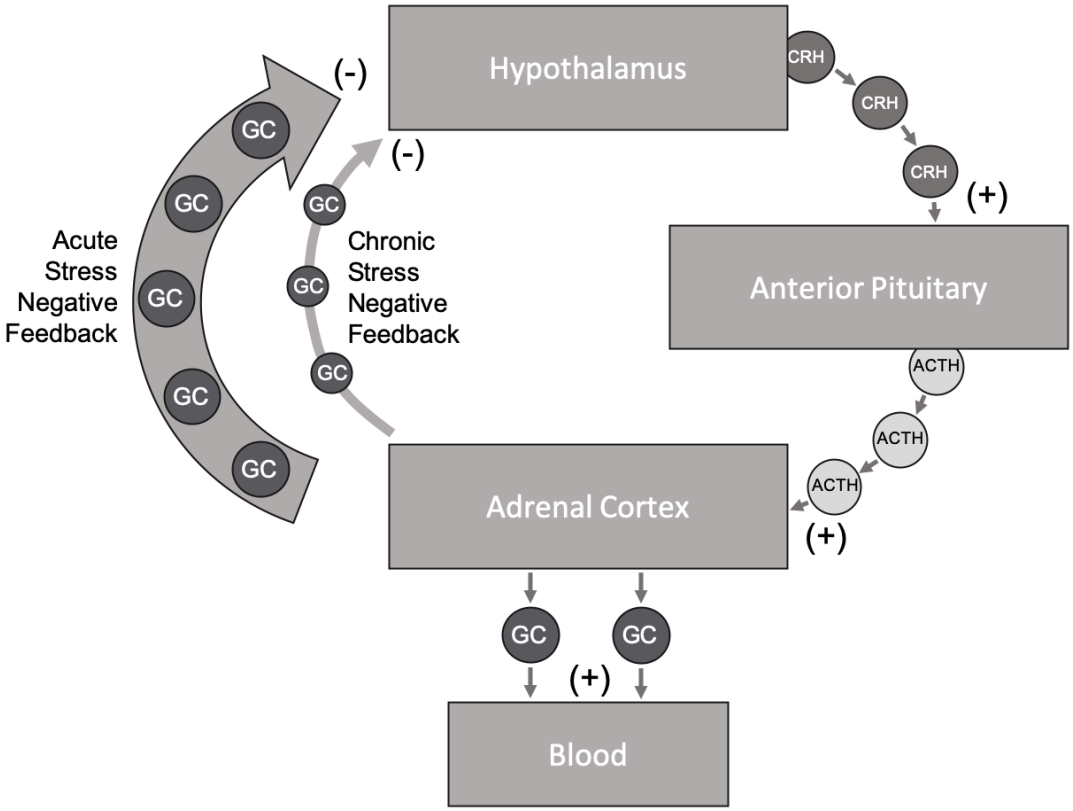

Figure 1. A visualization of the hypothalamic-pituitary-adrenal axis and corresponding hormones. Corticotropin-releasing hormone (CRH) is released from the hypothalamus, stimulating the pituitary to release adrenocorticotropic hormone (ACTH), which stimulates the adrenal cortex to release a category of stress hormones, glucocorticoids (GC). The negative feedback cycle of the HPA axis has an inhibitory effect on the hypothalamus in the presence of GC, returning circulating GC concentrations back to basal levels. Under chronic stress, the negative feedback cycle is diminished, generally maintaining high circulating GC concentrations.

Activation of the stress response increases an organism's chance of survival in the presence of a stressor (Carrasco and Van de Kar 2003). Two pathways are stimulated, allowing the organism to respond to both immediate and long-term threats.

Within seconds of exposure, the sympathetic nervous system responsible for the "fight-or-flight" response (Jansen and others 1995) releases catecholamines (predominantly epinephrine and norepinephrine) into the blood stream (Romero and Butler 2007). This increases heart rate, blood flow to the brain, and visual acuity, increasing an animal's overall alertness and capacity to respond to an acute threat (Reeder and Kramer 2005; Romero and Butler 2007). Concurrently, the HPA axis (Figure 1) is initiated; this is the slower and more enduring component of the stress response (De Kloet and others 1998). In the HPA axis, the hippocampus stimulates the paraventricular nucleus of the hypothalamus, causing the hypothalamus to release corticotropin-releasing hormones (CRH) into the hypophyseal portal system (Tsigos and Chrousos 2002; Reeder and Kramer 2005; Sheriff and others 2011). CRH is transported to the anterior pituitary, causing it to release adrenocorticotropic hormone (ACTH) into the blood stream (Sapolsky 2002). The presence of ACTH stimulates the adrenal cortex to secrete GCs (Carrasco and Van de Kar 2003). An increase in circulating GC levels are detectable within 3-5 minutes of the initial stressor, peaking within 15-30 minutes depending on the metabolic rate of the species (Möstl and Palme 2002; Sapolsky 2002; Sheriff and others 2011).

Under an acute stress event, an appropriate stress response can be life-saving. In the presence of a predator, for example, releasing catecholamines from the sympathetic nervous system allows an animal to respond within seconds by escaping or fending off the predator (Reeder and Kramer 2005). With elevated GC levels, the animal can cope

with the consequences of this interaction for minutes to hours afterwards (Sapolsky and others 2000). This helps the prey while being pursued or while hiding from nearby danger. A rush of GCs reallocates usable energy for mobilization (Sapolsky and others 2000; Sapolsky 2002; Hill and others 2012). Vasoconstriction supplies the muscles with glucose and oxygen, raising blood pressure and heart rate (Sapolsky and others 2000; Hill and others 2012). While blood flow increases to parts of the body that facilitate movement, it is reduced to the digestive system in an effort to reapportion energy to priority systems (Sapolsky 2002). GCs also suppress the release of several hormones involved in reproduction (luteinizing hormone, follicle-stimulating hormone, gonadotropin-releasing hormone, thyrotropin, and growth hormone), temporarily curtailing reproductive behavior (Sapolsky 2002; Hill and others 2012). Elevated GC levels alter the immune response by having an anti-inflammatory effect, potentially helping an injured animal retain mobility of an injured limb (Sorrells and others 2009). All these responses allow an animal to evade a predator, find refuge, avoid further detection, and heal from any physical contact.

A negative feedback cycle within the HPA axis (Figure 1), triggered by the presence of GCs above basal levels, down-regulates the production of additional GCs (Munck and others 1984). This negative feedback cycle uses glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), each with a different affinity for glucocorticoids (De Kloet and others 1998). GRs, predominantly found in the paraventricular nucleus, have a relatively low affinity for GCs compared to MRs, which

are most common in the hippocampus (Dallman and others 1987; Herman and others 2012). At low GC concentrations, MRs are mostly bound with GCs because MRs play a large role in the basal secretion and normal daily fluctuation of GCs (Herman and others 2012). GRs begin binding with GCs at concentrations typically seen during stress events (Herman and others 2012). During a stress-related spike in GC concentrations, the few remaining unbound MRs in the hippocampus bind to GCs, slightly inhibiting the signals sent from the hippocampus to the paraventricular nucleus (Dallman and others 1992). When the MRs are saturated, the GRs begin binding with GCs, creating an even stronger inhibitory effect on the paraventricular nucleus (de Kloet 1995; Herman and others 2012). The downstream effect of this process reduces the release of ACTH by the anterior pituitary. This negative feedback cycle allows the body to metabolize circulating GCs and return them to basal GC levels after exposure to acute stress (Carrasco and Van de Kar 2003). Chronic stress reduces the feedback cycle because signals from the hippocampus remain high, causing the hypothalamus to continue releasing CRH, resulting in a consistently high output of GCs (Boonstra and others 1998).

Circulating GC concentrations remain high during prolonged or repeated activation of the HPA axis (Sapolsky 2002). Most stress-altered processes are inhibitory in nature; therefore, when these processes are chronically activated they can reduce fitness (Romero and Wikelski 2001; Busch and Hayward 2009; Scarlata and others 2012; Wilkening and Ray 2016). A reduced reproductive drive can help an animal focus on a present danger, but chronically it reduces reproductive success (Breuner and others

2008). Under normal circumstances gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus into the hypophyseal portal system where it triggers the release of follicle stimulating hormone and luteinizing hormone from the anterior pituitary (Tilbrook and others 2002). The surge in production and release of luteinizing hormone and follicle stimulating hormone control reproductive functions such as estradiol production, follicular growth, ovulation, and the release of sex steroid hormones (Dobson and Smith 2000; Wingfield and Sapolsky 2003). Proper follicle growth and ovulation require a precise pulsatory release of GnRH and luteinizing hormone (Dobson and Smith 2000). If the timing, frequency, or magnitude of that release is disrupted, it can interfere with ovulation, estrous, and fertilization (Brann and Mahesh 1991; Fortune 1994; Dobson and Smith 2000). Chronically activating the HPA axis reduces the production and release of GnRH, harming reproduction (Tilbrook and others 2000). For example, captive Pygmy Rabbits (*Brachylagus idahoensis*) with high GC concentrations have a decreased ability to conceive, lowering their reproductive success (Scarlata and others 2012).

The metabolic response to stress is carried out primarily in the expression and resistance to insulin. Insulin is secreted from the pancreas and has many effects on metabolism (Andrews and Walker 1999). In adipose tissue, insulin facilitates glucose uptake, decreases the rate of lipolysis, and assists triglyceride uptake (Dimitriadisa and others 2011). In muscles, it increases glucose uptake, glycogen production, protein synthesis, and reduces the breakdown of glycogen and proteins (Sapolsky 2002;

Dimitriadisa and others 2011). In the liver, insulin promotes glycogen synthesis, reduces glycogen breakdown, and limits gluconeogenesis (Shimazu and Ogasawara 1975; Sapolsky 2002). This allows energy to be stored in adipose tissue and reduces excess circulating glucose. The influx of GCs and catecholamines decreases insulin secretion (Munck and others 1984), arresting the storage of glucose and other substrates in adipose tissue (Dimitriadisa and others 2011). It also encourages lipolysis, resulting in a rush of glucose and fatty acids into the blood stream where they can be utilized by exercising muscle (Sapolsky 2002; Tsigos and Chrousos 2002). The uptake of glucose in non-exercising muscle remains unchanged, allowing the maximum amount of energy to be redirected to parts of the body that help an animal flee a stressor (Sapolsky and others 2000). Chronic activation of the HPA axis reduces insulin production (Sapolsky and others 2000). This depletes adipose stores, deteriorates non-exercising muscle, and lowers insulin resistance, leading to diabetes and other metabolic diseases (Reeder and Kramer 2005).

Chronis stress alters many stages of digestion, beginning with reducing appetite (González-Torres and dos Santos 2019). Stress also disrupts the motor functions of the gastrointestinal (GI) tract (Taché and others 2001). GCs inhibit the release of gastric acid (Taché and others 2018), decrease motility of the upper GI, and increase motility of the lower GI (Garrick and others 1988; Taché and others 1993). High levels of GCs increase GI permeability, inflaming the intestines (Meddings and Swain 2000). Intestinal complications from chronic stress can lead to numerous gastrointestinal diseases and

compromise the immune response (Dhabhar and McEwen 1997; Mayer 2000; Sorrells and others 2009).

An individual's basal hormone levels and their reactivity to stressors can be influenced by life experiences. Different life stages (age, sex, reproductive status) affect basal GC concentrations (Romero 2002; Creel and others 2013; Wilkening and others 2013). Exposure to stress during development can alter the stress response later in life (Bonier and others 2009; Heim and others 2009). For example, some studies show that prenatal exposure to stress alters the HPA axis and these changes can be carried into adulthood (Welberg and Seckl 2001; Thayer and others 2018). However, researchers have not found a consistent reaction to prenatal stress exposure. Adults exposed prenatally may display reduced (Seckl 2004) or elevated (McCormick and others 2017) HPA responsiveness and increased or decreased basal GC levels (Kapoor and others 2008). This disparity likely stems from differences among stress intensity, frequency, duration, type, and exposure time (during gestation vs. after birth); and whether basal GCs or HPA responsiveness was tested (Glover and others 2010).

Chronic stress exposure can also lead to habituation, where the perception of a stimulus is changed and is no longer considered a stressor (Walker and others 2006). In the absence of habituation, chronic stress can cause physiological desensitization of the stress response, lowering the production of basal GCs and reducing the reactivity of the stress response resulting in overall lowered GC concentrations (Rich and Romero 2005; Cyr and Romero 2009).

The analysis and interpretation of stress hormones is inherently comparative; a single value of hormone concentration does not provide any information about the stress response. Interspecific basal levels and responses to stressors are highly variable (Millspaugh and Washburn 2004). For example, basal GC levels range from 5 ng/ml in Texas Horned Lizards (*Phrynosoma cornutum*) to 1270 ng/ml in Small flying foxes (*Pteropus hypomelanus*) (Busch and Hayward 2009). These numbers alone do not indicate that 1 species is more or less stressed than the other. Concentrations are not comparable among geographic regions and could lead to misinterpreting results (Wilkening and others 2016). Only GC concentrations from conspecific animals with similar climatic exposure and within the same geographic region should be compared (Palme 2005).

Hormones can be extracted from blood, saliva, tissue, hair, and feces (Sheriff and others 2011). Blood and saliva show the concentrations of circulating GCs at the time of sampling (Sheriff and others 2010). This is useful because the circulating GCs interact with tissue and promote the physiological changes in the body. Researchers measuring GC concentrations in plasma and saliva can quantify basal hormone levels, as well as an animal's response to a stimulus (Mormède and others 2007). This technique is invasive, requiring researchers to physically handle the animal to draw blood or swab the animal's mouth. Procuring a sample is stressful and this technique risks tainting the sample with hormones released in response to the capture event. GCs are detectable in blood and saliva 2-3 minutes after trapping (Romero and Romero 2002), making this

technique useful in laboratory settings but impractical in most field studies (Palme 2005).

Circulating GCs are metabolized and deposited in feces as fecal glucocorticoid metabolites (fGCM) (Palme 2018). Analyzing fGCMs offers a longer-term view of the average circulating GC levels (Millspaugh and Washburn 2004). This technique avoids measuring hormone peaks, providing a chronic view of hormones accumulated in the gut (Sheriff and others 2010). Fecal sampling shows chronic stress over the course of hours to days, depending on the metabolic rate of the species (Touma and Palme 2005). However, environmental conditions such as precipitation, exposure to sunlight, humidity, and freeze-thaw cycles can alter fGCM concentrations in deposited feces (Washburn and Millspaugh 2002; Wilkening and others 2016). These changes are unpredictable (Parikh and others 2018), requiring researchers to validate the collection procedure prior to sampling (Wilkening and others 2015). Some species are more readily used than others as model organisms in stress research. Species that are highly susceptible to environmental change can become indicator species for climate change or the effects of other human interference. For example, many species of pikas exhibit a stress response to environmental change such as losing sub-surface ice features (Wilkening and others 2015) or changing predation pressures (Yin and others 2017).

*American Pika (*Ochotona princeps*) Life History*

Pikas (*Ochotona*) are the only genus of the family Ochotonidae, which is restricted to the Northern Hemisphere (Smith and others 1990). North America is home to 2 pika species (Broadbooks 1965). The Collared Pika (*O. collaris*), occurs in Alaska, Yukon, Northwest Territories, and northern British Columbia (Smith and others 1990). The American Pika (*O. princeps*, hereafter referred to as "pika") is distributed in alpine and subalpine terrain of western North America. Their range begins in the north in central British Columbia, extending south to the Rocky Mountains of Arizona and New Mexico (Hafner and Smith 2010).

Pikas live in piles of large rocks, often found on slopes below rocky cliffs (Rapp and Fairbridge 1968; Smith and Weston 1990). Rocks in suitable pika habitat range in size from 0.2 m to 1.0 m in diameter (Tyser 1980). Pikas have been occasionally observed in piles of wood and scrap metal (Broadbooks 1965; Lutton 1975), but generally they are a talus-obligate species. They do not dig burrows; instead they use a network of openings between rocks below the talus surface (Lutton 1975). Pikas lack any means of active defense against predators (Smith and Weston 1990), but the talus provides refuge from Coyotes (*Canis latrans*), Long-tailed Weasels (*Mustela frenata*), Short-tailed Weasels (*Mustela erminea*), American Martens (*Martes americana*), and hawks (*Buteo* spp.) (Ivins and Smith 1983; Hafner and Smith 2010). Pikas are behavioral thermoregulators, using the cool subsurface temperatures offered by deep talus to lower their body temperature (Smith and others 2018). Their high basal metabolic rate

and high body temperature (average 40.1°C), coupled with a reduced ability to thermoregulate physiologically, contribute to pikas dying of hyperthermia when exposed to temperatures above 26-28°C for several hours (MacArthur and Wang 1973; Smith 1974).

Pikas give birth and rear their young within the safety of the talus (Millar 1974). Typically, females produce 2 litters per year consisting of 2 to 3 offspring per litter (Millar 1973; Smith and others 1990). Each year the average female will successfully wean only 1 litter (Smith and Ivins 1983). Juvenile pikas are weaned at 3 to 4 weeks, after which they disperse to seek out their own territories (Millar 1974). Juveniles often claim available territories close to their natal ones, but on rare occasions they are capable of dispersing nearly 2 km to find available habitat (Peacock 1994). Pikas are the embodiment of metapopulation theory (Moilanen and others 1998) because they colonize talus patches with limited connectivity. Together, these patches establish a greater metapopulation. Occasionally, juvenile pikas disperse from patches of high productivity (sources) to patches of adequate or substandard quality (sinks) (Kreuzer and Huntly 2003). The disparate birth rates of these source and sink patches allow patch occupancy to fluctuate even as the health of the overall metapopulation remains intact (Smith and Gilpin 1997).

Pikas forage by grazing and haying vegetation growing at the talus edge. If they venture too far away from the safety of the talus, they expose themselves to potential predation (Holmes 1991). Grazing is the direct consumption of vegetation, usually from

vegetation closest to the talus edge (Smith and Weston 1990), while haying is the act of collecting and storing vegetation in haypiles. Pikas will forage further from the talus when haying to collect desirable vegetation (Huntly and others 1986). Pikas do not hibernate (Dearing 1997a); instead they remain active during the winter months in the talus under a protective layer of snow, which insulates them from dangerously cold temperatures (Hafner 1993). Under the snow, they feed mostly from the haypile they amassed the previous growing season (Morrison and others 2009). Their foraging preferences are adapted to the regionally available vegetation (Smith and Erb 2013; Varner and Dearing 2014). Generally, pikas select more forbs and woody plants when haying than when grazing (Huntly and others 1986). They also select plants with high concentrations of secondary compounds (Dearing 1996) that are preserved better in haypiles, allowing pikas to maintain a high nutrient content in their cache throughout the winter (Dearing 1997b).

Pikas build haypiles within an established territory, which they defend from dispersing pikas (Lutton 1975). They oust rivals through aggressive chases and fights (Svendsen 1979), but direct conflicts are rare and usually between same-sex individuals (Smith and Weston 1990). Neighboring territories are frequently occupied by pikas of the opposite sex, often overlapping (Svendsen 1979; Smith and Ivins 1984). Pikas usually use less aggressive tactics in territorial displays. Cheek glands 5 to 10 mm below the ear allow pikas to mark and reinforce their territory boundaries (Meaney 1987). Pikas use vocalizations to demarcate territories, repel intruding conspecifics, and attract mates

(Conner 1982; Conner 1985a; Trefry and Hik 2010). They also vocalize to alert neighboring pikas of potential danger from predators (Conner 1985b).

Each pika has at least 1 haypile and multiple latrines (Broadbooks 1965). Latrines are created through the accumulation of fecal pellets in a particular spot within a territory (Nichols 2010). Pikas, like most lagomorphs, produce both cecal and fecal pellets. Cecal pellets (also called cecotropes) are rich in nutrients and have a high water content (Pehrson 1983; Smith and Weston 1990). Pikas practice cecotrophy by ingesting their cecotropes directly from the anus (Krear 1965; Pehrson 1983). Reabsorption of the cecal pellet's water and nutrients allows them to extract resources from their environment more efficiently (Alexander 1993). Fecal pellets are harder, drier, and can persist in the environment for over 35 years (Nichols 2010).

Pikas are a good model organism for studies of environmental stress because they have low mobility, individuals can be identified in the field, and they defecate in easily-identified latrines, making collection of fecal pellets possible. Our study is concerned with pika stress along an interstate highway, and how that may affect their fitness in that habitat. By understanding pika stress and the possible repercussions on fitness, we might discern how successful pikas may be at colonizing habitats near and within WCSs.

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CHAPTER II

JOURNAL ARTICLE

ARE HIGHWAYS STRESSFUL FOR PIKAS?: ANALYSIS OF STRESS HORMONES OF THE AMERICAN PIKA (*Ochotona princeps*) LIVING ADJACENT TO INTERSTATE 90 IN THE CASCADE RANGE OF WASHINGTON STATE

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ABSTRACT—Roads often disrupt ecosystem connectivity, harming many wildlife populations. Wildlife crossing structures perforate the barrier-effect of roads, providing safe passage for species dispersing across roads, thereby reconnecting ecosystems. Some wildlife species live in modified habitats along roads and in habitat features within

wildlife crossing structures, but their fitness in these potentially stressful environments is poorly understood. Chronic stress alters behavior, reduces reproduction, and has been linked to reduced survival. Animals affected by chronic stress in road-adjacent habitats could reduce the ability of wildlife crossing structures to improve connectivity across roads. I assessed stress hormone concentrations in a low-mobility species, the American Pika (*Ochotona princeps*), living in anthropogenic rock embankment along Interstate 90 (highway) in the Cascade Range of Washington. I extracted fecal glucocorticoid metabolite (fGCM) concentrations from fresh fecal samples to determine chronic stress levels in pikas living adjacent to the highway compared with those living in similar rock embankment in a rails-to-trails state park, and in natural rocky sites. I evaluated potential stressors in the highway and rails-to-trail habitats by measuring microclimate proxies (environmental temperatures, elevation, and longitude) and noise levels. A linear mixed-effects model was used to test for differences in fGCM concentrations among habitats and assess the potential effects of these environmental variables on stress. Pikas in the highway habitat had the lowest fGCM levels among the 3 habitats. Stress hormone concentrations showed significant relationships with longitude and noise, but not with elevation or mean daily temperatures. I hypothesize that the lowered fGCM levels of highway pikas may be a result of downregulation of the stress response under chronic exposure to stressors, potentially including highway noise. My results offer insights into the physiological response of pikas to potentially stressful environments disturbed by humans and may provide a good indicator of stress

levels expected for pikas colonizing wildlife crossing structures now being constructed to improve wildlife connectivity across I90.

Key Words: American Pika, *Ochotona princeps*, stress response, glucocorticoids, road ecology

INTRODUCTION

Ecosystems function best with a high degree of connectivity (Olds and others 2012). Habitat fragmentation, the separation and isolation of habitats through a physical or psychological barrier (Fahrig 2003), disrupts connectivity (Crooks and others 2011). This can cause a drop in genetic variation within species and lead to inbreeding (Andersen and others 2004; O'Grady and others 2006; Alharbi & Petrovskii 2016). Roads contribute to habitat fragmentation in several ways (Rico and others 2007; Fahrig and Rytwinski 2009). Some wildlife species actively avoid roads (Ford and Fahrig 2008; McGregor and others 2008). Others are attracted to new habitats along roadsides or cross roads during foraging and seasonal movements but are killed in wildlife-vehicle collisions, causing potentially harmful reductions in population size (Fahrig and Rytwinski 2009). Road-effect zones, areas away from the road edge that are ecologically affected by roads, disrupt connectivity by creating suboptimal habitats that reduce reproduction and survival (Eigenbrod and others 2009). In these zones, roads disrupt erosion and hydrological processes, as well as contribute excess light, noise, and chemical pollution to habitats next to roadways (Forman and Alexander 1998). This type of altered habitat creates stressful environments that are potentially harmful to fitness (Bonier and others 2009; Navarro-Castilla and others 2014).

Some of the negative effects of roads on wildlife can be ameliorated with wildlife crossing structures (WCS), which increase connectivity and reduce wildlife-vehicle

collisions by providing safe passage across roadways (Beier and others 2008; Huijser and others 2008). The success of WCS is often assessed from their frequency of use and, more recently, from genetic connectivity (Sawaya and others 2014; Soanes and others 2018) of populations, but few studies consider success in terms of individual fitness (Clevenger and Waltho 2000; D'Amico and others 2015). Habitats near (and within) WCS are inherently close to roadways and, therefore, within the potentially stressful road-effect zone where fitness can be reduced. Highly-mobile species can quickly disperse away from one of these stressful environments after crossing, but low-mobility species are likely to have much of their home range within the affected area. By analyzing stress hormones in low-mobility animals living in habitats along a highway, we can explore the fitness consequences of roadside habitats (Sapolsky and others 2000; Sheriff and others 2011).

Stress is an internal response to an external stimulus that is perceived as a threat to an individual (Sapolsky and others 2000; Sheriff and others 2011). Stress can be assessed by measuring glucocorticoids (GC), a category of hormones released during the stress response (Romero and Butler 2007). Stress activates the hypothalamic-pituitary-adrenal (HPA) axis. This stimulates the hypothalamus to release corticotropin-releasing hormone (CRH) to the anterior pituitary (Carrasco and Van de Kar 2003), which releases adrenocorticotropic hormone (ACTH) into the blood stream (Aguilera and others 2001; Dallman and Bhatnagar 2001). ACTH triggers the adrenal cortex to release GCs into the blood where they have targeted effects on tissue (Tsigos and Chrousos 2002). A

properly functioning stress response evolved as a method of coping with acute environmental challenges that could result in mortality (Romero and Wikelski 2001; Jessop and others 2013). However, chronic stress is detrimental to an individual's fitness, lowering reproductive output (Bonier and others 2009; Wilkening and Ray 2016). Exposure to chronic stress may increase basal production of GCs (Moore and others 1991; Fowler and others 1995) or can lower expected GC concentrations (Rich and Romero 2005; Schöpper and others 2012) caused by habituation to stimuli or through physiological desensitization of the stress response (Cyr and Romero 2009).

We chose the American Pika (*Ochotona princeps*) as our model organism to investigate stress levels of a low-mobility species living adjacent to roads. Pikas are habitat specialists with small home ranges and limited dispersal capabilities, and they are subject to environmental stress such as extreme temperatures (MacArthur and Wang 1973; Wilkening and others 2015). These small lagomorphs are distributed discontinuously across the mountains of western North America (Smith and Weston 1990). As talus specialists, they live almost exclusively in piles of deep rock (Smith 1974), but can also occupy anthropogenic habitat patches. In the Cascade Range of Washington, pikas colonize rock embankments that stabilize the shoulders of an interstate highway (Ernest K, personal communication, multiple occasions). We hypothesized that the persistent stressors of a major highway could elicit a chronic stress response.

Our first objective was to compare chronic stress levels of pikas, as measured by fGCM concentrations, between habitats adjacent to an interstate highway and habitats farther from the highway environment. We predicted that in the absence of habituation or downregulation, pikas within the interstate highway habitat would have higher fGCM concentrations than pikas from the other habitats. Our second objective was to identify some possible stressors that may contribute to altered fGCM levels. A better understanding of the physiological impacts of major roadways on low-mobility animals can contribute to improved WCS design that might lower wildlife stress and more effectively increase connectivity.

METHODS

Study Area

We conducted this study on the eastern slope of the Cascade Range in central Washington (**Error! Reference source not found.**). The rain shadow of the Cascade Range creates an increasingly drier climate east of the crest. Average annual precipitation ranges from 254 cm at Snoqualmie Pass (elev. 900 m), the western boundary of this climatic region (WRCCa), to 132 cm at the southern shore of Lake Kachess, 24 km to the east (elev. 692 m) (WRCCb). This region is dominated by a mixed-

coniferous forest which comprises Douglas-Fir (*Pseudotsuga menziesii*), Western Redcedar (*Thuja plicata*), Western Hemlock (*Tsuga heterophylla*), Pacific Silver Fir (*Abies amabilis*), and Western White Pine (*Pinus monticola*). Species often found in the understory include Vine Maple (*Acer circinatum*), Devil's Club (*Oplopanax horridus*), Western Skunk Cabbage (*Lysichiton americanus*), Bracken Fern (Pteridium aquilinum), and horsetail (*Equisetum* sp.). Broad-leaf shrubs and trees, such as willow (*Salix* sp.), alder (*Alnus* sp.), and cottonwood (*Populus* sp.) are found closer to wet areas (Kovalchik and Clausnitzer 2004).

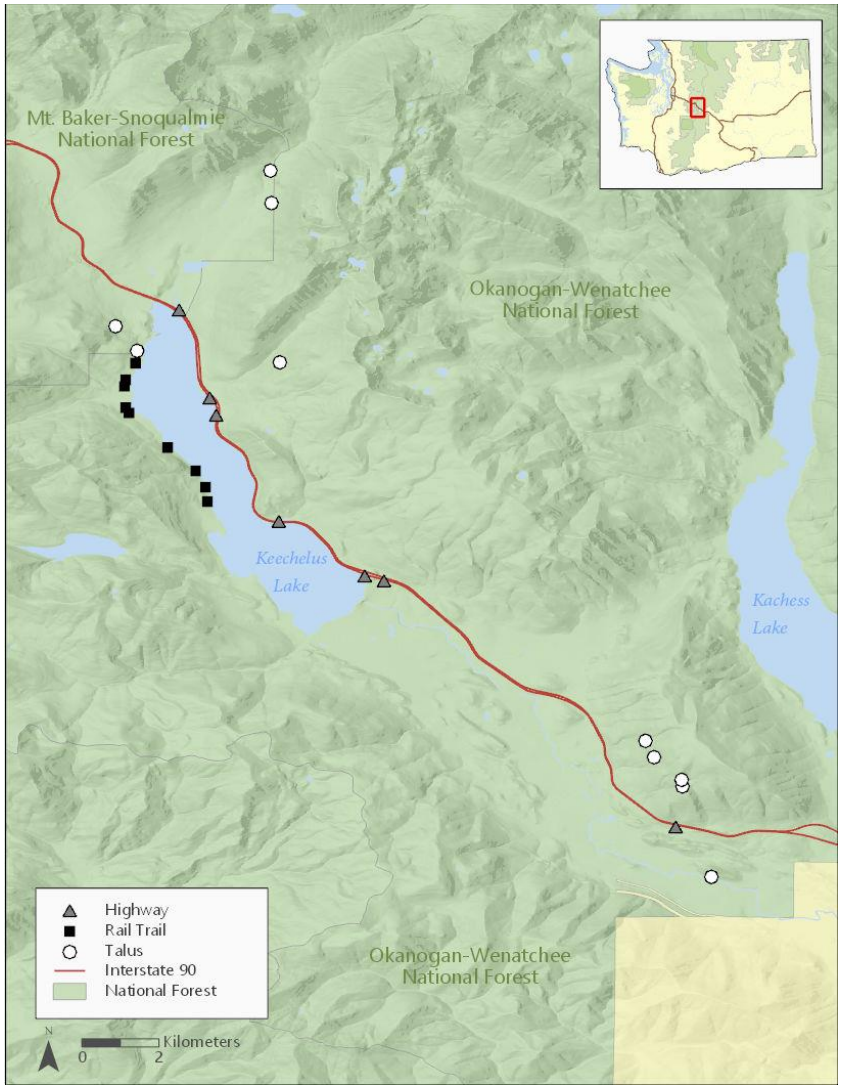

Figure 2. Fecal pellet collection sites within the Snoqualmie Pass East Project area of the Interstate 90 transportation corridor. Pellets were collected from 3 habitat types: Highway rock embankment (Highway), Palouse-to-Cascades Trail rock embankment (Rail Trail), and natural talus (Talus).

Within this study area, a major east-west interstate highway (I90) descends from Snoqualmie Pass and runs along a valley floor flanked by mountains with natural talus slopes. I90 hosts an average 31,000 vehicles per day traveling across Snoqualmie Pass

(WSDOT). A non-motorized recreational trail (Palouse-to-Cascades Trail) converted from a former railroad line, runs parallel to I90 at a distance of 1-2 km. Multiple wildlife crossing structures are being incorporated into I90 within the I90 Snoqualmie Pass East project area from Hyak to Easton to increase ecological connectivity in the Cascade Range (WSDOT 2008; Long and others 2012). Our study area overlaps the WSDOT project area to draw comparisons between existing highway-adjacent habitat and the habitat that will be available in WCS. Given the similar proximities to the road-edge, stressors in the highway-adjacent habitat likely will be the same stressors present in WCS. By evaluating stress hormone concentrations in highway-adjacent habitat, we can reasonably predict the expected hormonal response of individuals in habitat within and surrounding WCS.

We compared 3 distinct pika habitat types in the study area: rock embankment adjacent to the I90 shoulder ("Highway"), rock embankment along the shoulders of the Palouse-to-Cascades Trail ("Rail Trail"), and natural talus or scree fields on hillsides surrounding the I90 transportation corridor ("Talus"). We chose these 3 habitat types (Highway, Rail Trail, and Talus) because they represent decreasing levels of human disturbance (and presumably stress) from Highway to Rail Trail to Talus. All Highway sites were within 6 to 70 m (20.5 m on average) of the highway edge, a distance we considered to be highly influenced by the interstate. The 2 sites farthest from the highway (50 and 70 m) both had clear lines-of-sight and minimal vegetation blocking their exposure to the highway. The Rail Trail has rock embankment similar to that along

I90 with similar rock size and type, slope, aspect, and hydrologic conditions. Talus occurs away from the influential effects of I90 and provides a baseline for the expected hormone concentration of pikas in our study.

Fecal Samples

We measured chronic stress by extracting fecal glucocorticoid metabolites (fGCM), the metabolized GCs deposited in feces (Sheriff and others 2011). During digestion, baseline and peak levels of circulating GCs are essentially averaged as they are excreted as fGCMs. This provides a better assessment of chronic stress compared to measuring GCs in blood or saliva, which is best used to evaluate acute stress responses (Millspaugh and Washburn 2004; Wilkening and others 2016).

Sample Collection.—We determined chronic fGCM concentrations from fecal pellets collected from latrines within pika territories during a 4-week period in mid-summer, 2018 (Appendix A). We identified territories through occupancy surveys, behavioral cues, and haypile locations; and conservatively considered pikas with 50 m between active haypiles to be unique individuals (Broadbooks 1965; Smith 1974). A power analysis of a separate data set (Wilkening and others 2013) suggested a minimum n = 20 samples per habitat. We met or exceeded this target, with n = 20 from 7 Highway sites, n = 21 in 9 Rail Trail sites, and n = 23 in 11 Talus sites (Appendix A).

To ensure accurate fGCM measurements, we collected only fresh fecal pellets (Wilkening and others 2016; Parikh and others 2018), identified by their dark greenish color with some obvious pieces of vegetation and their superior position in the latrine (on top), often stuck together in small piles (Nichols 2010). Aging pellets dry out, turn brownish-grey, and fall loosely to the latrine floor (Jeffress and others 2011). Each sample contained a minimum of 15 fecal pellets per individual; when necessary, we collected pellets from multiple active latrines within an individual's territory. We placed pellets in a 1.5 mL microcentrifuge tube with a tight-fitting cap, then immediately placed the sample on ice in a cooler, until transport to a -20°C freezer for storage prior to extraction (Touma and Palme 2005; Wilkening and others 2013). To avoid contamination, we changed gloves after collecting from each individual and cleaned all equipment that contacted fecal pellets with 90% ethanol. Techniques involving pikas were approved by the Institutional Animal Care and Use Committee of Central Washington University (Protocol #A091601) and the Washington Department of Fish and Wildlife (Scientific Collection Permit #ERNEST 18-190).

Fecal Glucocorticoid Metabolite Analysis.—To standardize our laboratory technique, all fecal extractions were performed by Thomas McIntyre at Central Washington University using a commercially available DetectX® Corticosterone Enzyme Immunoassay (EIA) kit from Arbor Assays, Inc (Ann Arbor, MI; catalogue number K014-H1). We followed the Arbor Assay Steroid Solid Extraction Protocol, which was previously validated for pika fGCM (Wilkening and others 2013). Pellets were lyophilized

and powdered with a mortar and pestle. We added 100% undenatured ethanol (1 mL per 0.1 g feces), mixed the solution on a vortex mixer (Daigger Vortex Genie 2; Daigger Scientific, Inc., Vernon Hills, IL) for 30 minutes, then centrifuged samples at 5,000 rpm for 15 minutes. The supernatant was pipetted off the pellet then evaporated in a vacuum centrifuge (Thermo Savant SC110A Plus with a UVS400 Universal Vacuum System; Savant Instruments, Inc., Farmingdale, NY). The fully dried supernatant was stored at -20°C prior to EIA analysis.

Before performing the assay, the supernatant was reconstituted with an assay buffer supplied in the kit. All samples were randomized when transferred to a microplate and were run in triplicate and compared against a standard curve of 7 known corticosterone concentrations (5000, 2500, 1250, 625, 312.5, 156.25, and 78.125 pg/mL). The fGCM concentration of our samples was determined by reading the optical density via a microplate reader (BioTek μQuant Microplate Reader and BioTek Synergy 2 Microplate Reader; BioTek Instruments, Inc., Winooski, Vermont) with Gen5 1.11 Data Analysis software. Intra-assay coefficients of variation were < 10% and inter-assay coefficients of variation were < 15%. The final concentrations of fGCM were reported as picograms per gram of dried feces.

Environmental Stressors

We measured stimuli that have been identified in previous studies as stressors across a variety of species (Hansen 2009; Wilkening and others 2011; Takahashi 2012; Kleist and others 2018) and are relevant for pikas. We focused our sampling efforts on the Highway and Rail Trail habitats, which differ greatly in the extent of human disturbance (and presumed stressors), but are otherwise similar in elevational gradient, rock type and placement, and anthropogenic origin.

Microclimate.—Elevation and temperature are often used as proxies for microclimate in pika stress-hormone analysis (Wilkening and others 2015). Due to the intense drop in annual precipitation from west to east in our study area, we also considered longitude to be an important microclimate proxy. We recorded longitude and elevation at the center of each site using a handheld GPS device (Garmin GPSmap 76CSx; Garmin International, Inc., Olathe, Kansas).

We deployed data loggers at a subset of sites (5 Highway and 7 Rail Trail sites) to record subsurface temperatures. Since subsurface temperatures in rock piles can depend on air flow between rocks, we placed two data loggers per site: one HOBO data logger (UA-002-64 Onset HOBO; Onset Computer Corporation, Bourne, MA) and one Thermochron iButton data logger (DS1921G Maxim Integrated Products, Inc., San Jose, CA). Each was placed approximately 0.8 meters below the rock surface and within 10

meters of an active haypile, (Waterhouse and others 2017). Data loggers recorded temperature every hour for 7 days prior to fecal pellet collection.

Noise.—Pikas rely on vocalization for intraspecific communication (Conner 1985), and because noise has the potential to disrupt pika vocal communication and other auditory functions, we measured environmental sound levels. We expected traffic to be a major contributor to noise along the Highway habitat, so we collected sound measurements between 11:00 and 16:00, the hours of peak traffic (1800-2300 vehicles per hour) based on 2007-2017 average hourly traffic data (Josh Zylstra, WSDOT, 2018, unpublished data). We recorded sound levels with a class 1 sound level meter (SVAN 971; Svantek, Warsaw, Poland) at our Highway (n = 19) and Rail Trail (n = 21) sites (we did not record sound measurements at 1 site along Highway due to the additional presence of construction equipment). We mounted the meter 0.3 m above the surface of the rocks using a tripod. We recorded the equivalent continuous sound pressure level (Leq) over a 2-minute interval, identified as an acceptable sampling period (Hooper 2011; Kleist and others 2018). Leq is unweighted, providing an unfiltered assessment of sound pressure over all frequencies without confining our measurements to a species-specific hearing range. Sound was recorded when wind speeds were < 4 km/hr (Hooper 2011).

Predator Detection.—The high level of disturbance in the Highway habitat may deter predators from foraging in this area, which could contribute to lower stress. To detect potential predators and differences in predation pressure between habitats, we

used wildlife camera traps to document predator visitations to pika territories. We installed 8 camera traps in 7 sites along Highway (1 Reconyx HC600 HyperFire, 1 Reconyx RC60 RapidFire, 2 Reconyx Silent Image PM35M13, 1 Bushnell Trophy Cam 119447, and 3 Reconyx UltraFire) and 8 cameras in 8 sites along Rail Trail (1 Reconyx HC500 HyperFire, 1 Reconyx RC55 RapidFire, 2 Reconyx RC60 RapidFire, 2 Reconyx Silent Image PM35M13, and 2 Bushnell Trophy Cam 119447). Cameras were set to record 3 to 5 images when triggered. We focused each camera on an active haypile and placed it within 5 m of the haypile.

Data Analysis

We analyzed fGCM concentrations at all sites using a linear mixed-effects (LME) model to predict fGCM concentrations. Habitat, elevation, and longitude were used as fixed explanatory variables, and site was used as a random variable, accounting for the hierarchical structure of our data (Pinheiro and others 2019). We tested all residuals for normal distribution and homogeneity of variance with the *shapiro.test* and *leveneTest* functions, respectively, in R (R Core Team 2018; Fox and Weisberg 2019). Log transformations were performed where necessary to meet the assumptions of the models used.

Because we recorded temperature and sound only at Highway and Rail Trail habitats, we analyzed them separately. We extracted 3 derived temperature

measurements from the data loggers: mean daily temperature, number of days with a maximum temperature above 26°C, and number of days with a maximum temperature above 28°C (Wilkening and others 2011; Beever and others 2010). During data exploration, average daily temperature, average number of days above 26°C, and average number of days above 28°C per site were each used as a predictor variable independently in different analyses. We retained only average temperature in our model given the lack of predictive power from the other variables. We fit a linear model (LM) with temperature as the predictor variable and mean fGCM concentrations per site as the response variable. We used a LM because we had only 1 measurement per site. We also used a Welch's two-sample t-test on mean daily temperature between the 2 habitat types to determine if temperatures differed between Highway and Rail Trail sites. We compared fGCM concentrations (response variable) to sound (Leq, predictor variable) in a separate linear mixed-effects model. Due to high collinearity between Leq and habitat, we did not include habitat in this model.

We performed all analyses using the following packages of R version 3.5.2: *nlme* for linear mixed-effects models (Pinheiro and others 2019), *car* for Levene's Tests (Fox and Weisberg 2019), and *emmeans* as a post hoc analysis (Lenth 2019) to calculate the significance between pairs of habitat type (EMMs) (R Core Team 2018).

RESULTS

Fecal Glucocorticoid Metabolite Concentrations

Habitat type was a significant predictor of fGCM concentrations in pikas at our study sites (Table 1) (LME, $F = 9.532$, $P = 0.001$). Pikas living adjacent to Highway had significantly lower fGCM concentrations than pikas living in Rail Trail sites (EMMs, $P = 0.001$) and Talus (EMMs, $P = 0.002$) (Figure 3). Pikas from the Rail Trail and Talus habitats showed no difference in fGCM concentrations (EMMs, $P = 0.997$).

Table 1. Synopsis of models and results. The models are labeled based on the model type (LME = linear mixed-effects model, LM = linear model) and the main predictor variable used. Predictor variables are habitat type (Habitat) with 3 levels (Interstate 90 = Highway, Palouse-to-Cascades Trail = Rail Trail, and natural talus = Talus), elevation, and longitude of each rocky site where we collected fecal samples. Significant P-values ($P < 0.05$) are denoted with an asterisk.

Model	Response Variable	Predictor Variable	*df*	*F*	*P* - value
LME	log(fGCM)	Habitat	21	9.532	0.001*
		Highway - Talus	21		0.002*
		Highway - Rail Trail	21		0.001*
		Rail Trail - Talus	21		0.997
		Elevation	21	0.762	0.393
		Longitude	21	13.572	0.001*
LM - Temperature	fGCM	Habitat	1	1.855	0.206
		Mean Site Temperature	1	0.001	0.979
LME - Sound	log(fGCM)	Leq	23	6.454	0.018*

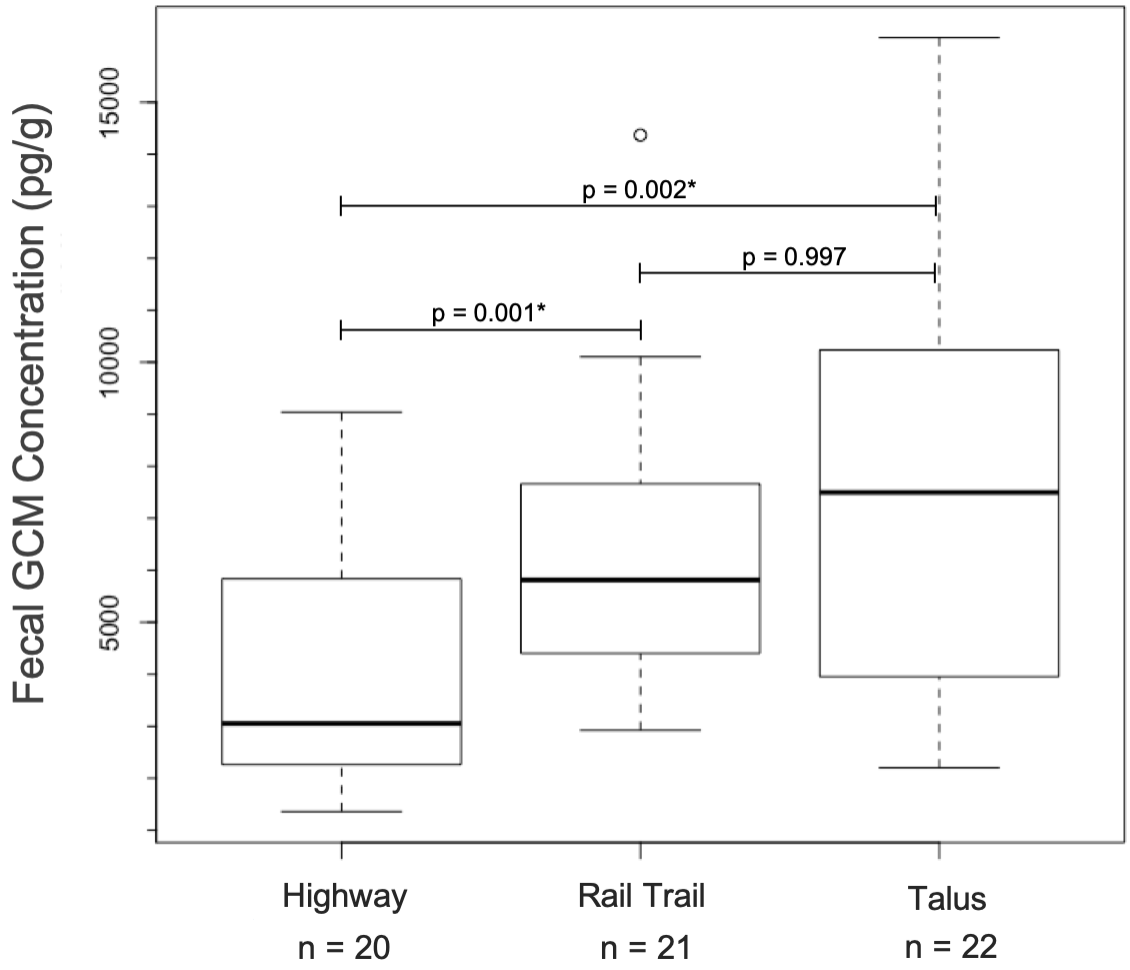

Figure 3. Fecal glucocorticoid metabolite (fGCM) concentrations, expressed as picograms fGCM per gram of dried feces, collected from 3 rocky patch habitats: Interstate 90 (Highway), Palouse-to-Cascades Trail (Rail Trail), and natural talus (Talus). Boxes indicate 25% and 75% quartiles, dark bars are median fGCM concentrations, and whiskers depict 95% confidence intervals.

We had too few samples per site to test for differences in variance among habitats while still including site as a random variable within our model. Consequently, we used a Levene's test for homogeneity of variance on fGCM concentration values

without accounting for the hierarchical structure of the data. This test showed significant heterogeneity among the 3 habitats (F = 3.788, P = 0.028). Pikas in natural talus showed the largest variation in fGCM concentrations of the habitats sampled (Figure 3).

Elevation was not a significant predictor of fGCM concentrations (LME, F = 0.762, P = 0.393). Mean elevation of the natural talus was the highest among the 3 habitats (Kruskal-Wallis rank sum test, $\chi 2$ = 26.4, P < 0.001) (Figure 4). Elevation was very similar at sites in Highway and Rail Trail habitats ($\bar{x}$ = 768.1 m, s = 1.7 and $\bar{x}$ = 776.1 m, s = 1.0, respectively).

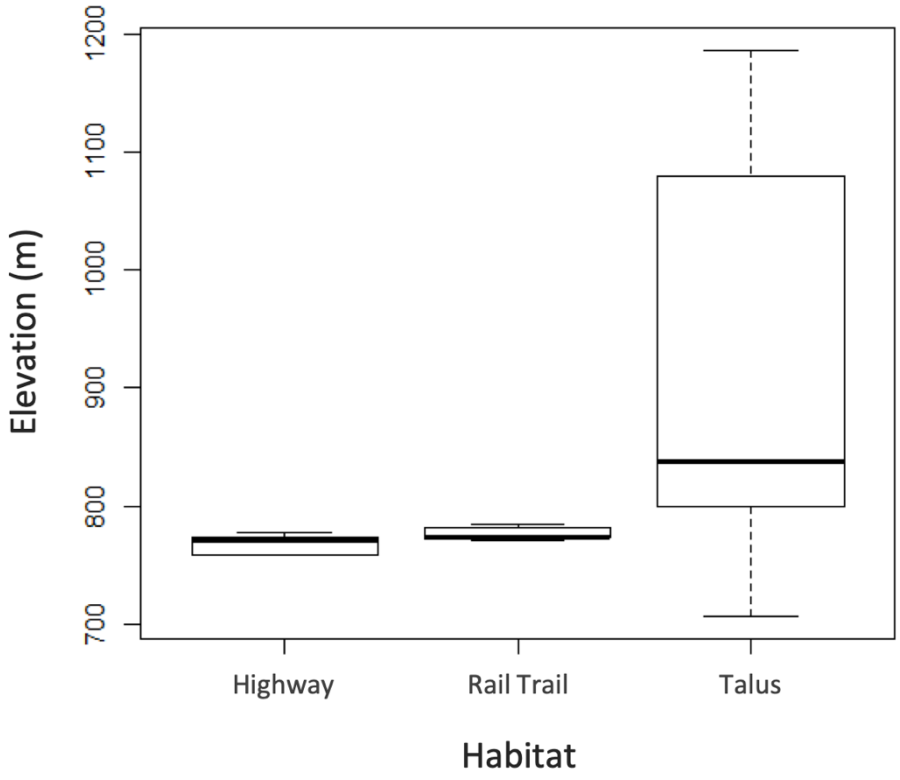

Figure 4. Elevation of sites from Interstate 90 (Highway), Palouse-to-Cascades Trail (Rail Trail), and natural talus (Talus) habitats. Natural talus had the highest mean elevation. Despite differences in elevation, no correlation was found between fGCM concentrations and elevation. Boxes indicate 25% and 75% quartiles, dark bars are median elevations, and whiskers depict 95% confidence intervals.

Longitude was significantly correlated with fGCM concentrations, with fGCMs increasing eastward (Figure 5) (LME, $F = 13.572$, $P = 0.001$). We did not detect any significance between longitude and habitat type during model selection, so we did not include the interaction term in our model.

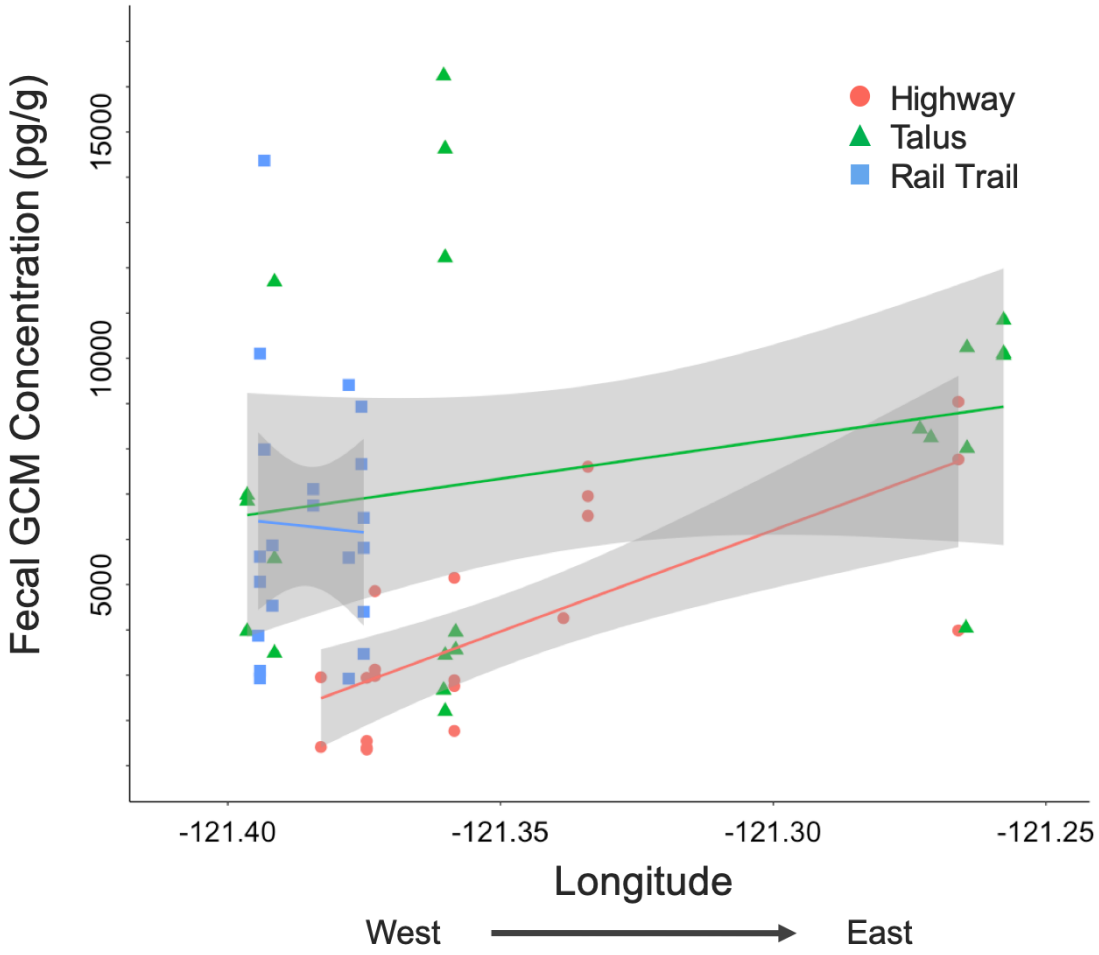

Figure 5. Pika fecal glucocorticoid metabolite concentrations plotted against the longitude (from west to east) of fecal pellet collection sites from 3 habitats: Interstate 90 (Highway), the Palouse-to-Cascades Trail (Rail Trail), and natural talus (Talus).

Average daily subsurface temperature did not predict fecal GCM concentrations (LM, $F = 0.001$, $P = 0.979$), and mean subsurface temperature did not differ between Highway and Rail Trail ($\bar{x} = 19.3$°C, $s = 0.6$ and $\bar{x} = 19.6$°C, $s = 1.0$, respectively; $t = -0.272$, $P = 0.791$).

Sound

Concentrations of fGCM were log-transformed to meet assumptions of normality. Mean sound levels at the Highway sites were significantly higher than those along the Rail Trail (Figure 6), $\bar{x}$ = 74.2 dB, s = 0.18 and $\bar{x}$ = 66.7 dB, s = 0.22, respectively (Welch's Two-Sample t-test; t = 5.916, $P < 0.001$). Leq (dB unweighted) was negatively correlated with fGCM concentrations (Figure 7; marginal R^2 = 0.188, conditional R^2 = 0.493, F = 6.454, P = 0.018).

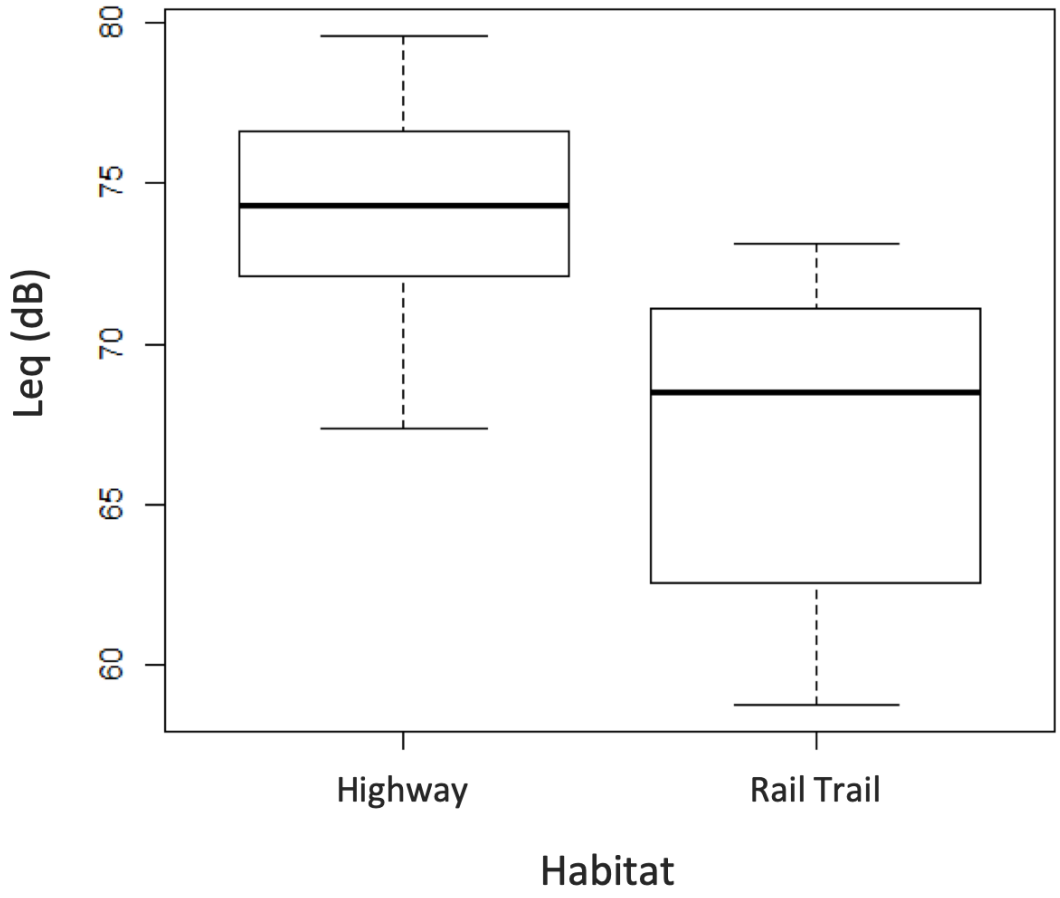

Figure 6. Sound pressure averaged over 2 min (Leq) recorded at times of peak traffic volume. Sound level was significantly louder along Interstate 90 (Highway) than along the Palouse-to-Cascades Trail (Rail Trail). Boxes indicate 25% and 75% quartiles, dark bars are median Leq (dB), and whiskers depict 95% confidence intervals.

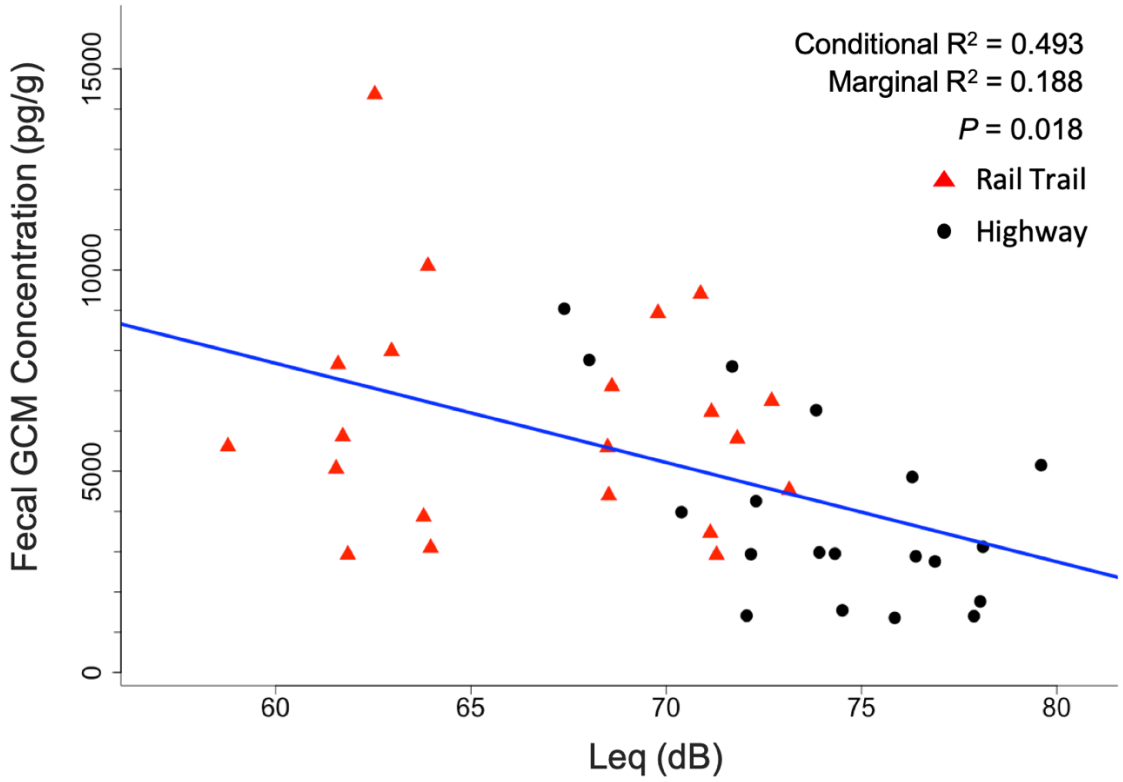

Figure 7. Relationship of fecal glucocorticoid metabolite (fGCM) concentration (pg/g) to sound pressure averaged over 2 min (Leq) for pikas along Interstate 90 (Highway) and Palouse-to-Cascades Trail (Rail Trail). All sound recordings were taken at sites of fecal collections.

Predators

Over the course of 719 camera trap-nights at Highway and Rail Trail habitats, we identified visitations from 16 different species (Appendix B). We were able to confirm the presence of 4 potential predator species (Figure 8). Highway sites were visited by 1 Bobcat (*Lynx rufus*), 1 Long-tailed Weasel (*Mustela frenata*), and 1 House cat (*Felis*

catus). We did not detect any natural predators in Rail Trail, although we did record 1 off-leash dog (*Canis lupus familiaris*).

Figure 8. Predators identified from camera traps visiting pika haypiles in highway-adjacent habitat (A, B, & C) and a haypile along the Palouse-to-Cascades Trail (D). Predators included: *Lynx rufus* (A), *Felis catus* (B), *Mustela frenata* (C), and *Canis lupus familiaris* (D).

DISCUSSION

In efforts to improve ecological connectivity, conservation ecologists often must link natural landscapes via less-than-ideal habitats. To appropriately assess the strength of these linkages, we must identify potential negative effects on species. Designers of wildlife crossing structures (WCSs) across highways incorporate a variety of habitat features that encourage animals to move through crossing structures, and also encourage lower-mobility species to colonize the structures. Attracting low- and high-mobility species offers a breadth of ecological connections, but places vital subsets of communities in potentially stressful environments for significant portions of their lifetimes. We can continue developing more successful efforts in habitat connectivity by identifying the effects of stressors on low-mobility species in roadside habitats.

In this study, our goal was to determine if stress hormone levels of pikas were different in habitat close to an interstate highway compared to more natural habitats. We found that pika stress hormone concentrations differed between habitats in the Cascade Range of central Washington. Contrary to our prediction, pika fGCM concentrations were lowest at sites along the Highway. Conversely, pikas living in Talus had the highest hormone concentrations. Hormone levels from the Rail Trail habitat were significantly higher than hormone levels from the Highway habitat ($P = 0.001$) but were similar to the Talus habitat ($P = 0.997$).

Interpreting Stress Hormones

GC concentrations can vary with age, sex, and breeding status in most species, including pikas (Wilkening and others 2013). The noninvasive nature of our sampling technique (collecting fecal samples without handling individuals) did not allow us to definitively assess life stage or breeding status, but by sampling within a short time-frame and using an appropriate sample size from each habitat, we strove to minimize the confounding effects of age, breeding status, and sex (Wilkening and others 2013; Brann and Mahesh 1991; Sapolsky and others 2000).

Stressful external stimuli can elevate GC concentrations, but chronic stress can have the opposite effect. Lowered hormone levels can result from a change in the way an animal psychologically or physiologically processes those stimuli (Palme 2005), and can be caused by exhaustion, habituation, or physiological downregulation of the stress response (Dallman and Bhatnagar 2001; Tilbrook and others 2002; Cyr and Romero 2009). Exhaustion is the inability of the body to continue producing hormones within the hypothalamic-pituitary-adrenal (HPA) axis due to overproduction (Selye 1946; Rich and Romero 2005). This mechanism is contentious in stress research as an explanation of chronic stress due to inconclusive evidence on whether components of the HPA axis can deplete the supply of hormones (Boonstra and others 1998; Sapolsky 2002).

The 2 most readily-testable causes of lowered stress hormones are habituation and physiological downregulation of the stress response (downregulation). Habituation

occurs when an animal's perception of a stimulus changes (Levine and others 1989). Habituation is therefore strongly rooted in psychology, rather than physiology (Cyr and Romero 2009). A stimulus that is novel, unpredictable, and out of the animal's control (Levine and others 1989) will be perceived as stressful. But once the stimulus loses these qualities, an animal will habituate to the stressor and will no longer trigger a stress response.

Downregulation decreases the responsiveness of the HPA axis as a way to adapt to the presence of a chronic stressor (Cyr and Romero 2009). This allows individuals to avoid the negative consequences to health associated with chronic stress by reducing the functionality of some component of the HPA axis (Cyr and Romero 2009; McEwen 2009). For example, in chronically-stressed European Starlings (*Sturnus vulgaris*), the physiological response to stress was changed rather than habituation through an alteration of the release of ACTH from the pituitary (Rich and Romero 2005). The concept of downregulation can be applied in other taxa, but the rate-limiting step is likely to be species-dependent (Cyr and Romero 2009; Schöpper and others 2012).

Habituation and downregulation can both result in lowered GC concentrations, but the mechanism that facilitates this reduction is different. Habituation applies to an animal's response to a stimulus. In the presence of a novel stressor, hormones spike (Sapolsky and others 2000). As an animal learns a particular stressor is not a threat, the stress response to that stimulus can gradually decrease, causing the hormonal peaks to become less severe (Dallman and Bhatnagar 2001). Eventually the stimulus evokes little

to no response from the animal, and hormone levels are maintained at their original baseline levels (Dubovicky and Jezova 2004; Grissom and others 2007; Cyr and Romero 2009). Downregulation, in contrast, reduces the HPA axis responsiveness, lowering production of hormones during a stress response and during the baseline, daily production of GCs (Rich and Romero 2005).

The most likely explanation for the lowered stress hormone concentrations in the Highway habitat in our study is that pikas are experiencing either habituation or downregulation. The majority of the stressors faced by the Highway pikas would likely lose their novelty over the course of their exposure. However, many of these stressors likely remain out of their control and are unavoidable. Pikas may become accustomed to vehicles in general, but the frequency and intensity of large, loud vehicles still remains unpredictable. The low mobility of pikas does not allow them to control their exposure to the roadside stressors. They are able to retreat below the talus, but if the stressors penetrate the rock surface this may not provide the refuge needed to give pikas the perception of control.

If the animals in our study were habituated to the stressors of the highway, we would expect to see the same fGCM concentrations between those individuals and pikas from the natural habitat. If pikas habituated to the Highway, fGCM concentrations (an average of the frequency and intensity of hormonal peaks and circulating baseline GCs) should be the same between the habitat where the stressor never existed (Talus) and the habitat where the stimuli are no longer perceived as stressors (Highway). Also, while

habituation essentially removes the response to a stressor, the natural GC variation among individuals is maintained. If the Highway pikas were habituated, they would show no difference from pikas at Talus sites in either average fGCM concentrations or degree of variation among individuals. This was not the case, so we suspect some other mechanism is causing the reduction of fGCM in the Highway habitat. The hormone reduction and the lower inter-individual variation in the Highway pikas, compared to the Talus pikas, is more consistent with downregulation or other processes of physiological desensitization to the stress response. An alternative explanation – that pikas along I90 are less stressed than pikas in the other habitats – cannot be ruled out, but is unlikely and not supported by our data on potential stressors.

Potential Stressors

Pikas are highly sensitive to temperature (Smith 1974; Smith and Weston 1990; Wilkening and others 2016). The consistent subsurface temperatures offered by talus provide a refuge from the potentially life-threatening highs and lows at the surface (Smith and Millar 2018). We used elevation, longitude, and subsurface temperature as proxies for microclimates that may have contributed to a more stressful environment. Across all three habitats, elevation was not a significant predictor of fGCM levels. This lack of correlation may have been due to similar elevations at Highway and Rail Trail sites (Figure 4). Elevation may relate to fGCM concentrations in other regions (Wilkening

and Ray 2016), but a relationship was not detected in our study system. While our sites do not cover the same longitudinal gradients in all 3 habitats (**Error! Reference source not found.**), longitude was significantly associated with fGCM concentrations. In our study area along the leeward slopes of the Cascade Range, annual precipitation decreases dramatically from west to east, which may explain why pikas from the eastern sites had higher stress hormone concentrations than pikas from the western sites (Figure 5).

Subsurface temperatures at Highway and Rail Trail sites were practically identical. We expected fGCM concentrations to have some relationship with subsurface temperatures. However, the extremely low variation in temperatures among sites was likely responsible for the lack of correlation between stress and subsurface temperatures. We report these results with caution; microclimates with more extreme temperatures may still cause stress in pikas. Surface temperature may also contribute to stress since pikas are active above the surface about 30% of daylight hours (Smith and others 2018). They take refuge under the surface, but rocky habitats buffer temperatures at different depths. The depth of the available habitat could affect a pika's ability to control temperature as a stressor.

Biologists are becoming increasingly aware of the effect that noise has on physiological stress in free-living wildlife (Blickley and others 2012; Mancera and others 2018). Traffic noise elicits a stress response in Wood Frogs (*Lithobates sylvaticus*) and can alter their mating behavior (Tennessen and others 2014). Species reliant on vocal

communication are particularly vulnerable to the stressful effects of noise (Shannon and others 2016). Vocalizations are a regular tool for communication among pikas in talus patches. Pikas rely on auditory cues from neighbors to alert them to potential predators (Conner 1985a). A variety of calls are used during the breeding season and to demarcate territories (Conner 1985b). We expected any threat to that communication system to cause some degree of stress (Hayes and Huntly 2005). We found an inverse relationship between stress hormone levels and sound levels. Pikas in quieter sites had higher fGCM concentrations than those in much louder locations (Figure 7). Because units for sound, dB, are on a logarithmic scale, volumes from Rail Trail sites were equivalent to between a whisper and conversational speech, while some Highway sites experienced sustained volumes reaching those of a freight train from 30 m away (OSHA 2013). The topography of the landscape protected some Highway sites by considerably dampening the sound before it reached the rocks. Other sites were far more exposed, subjecting those pikas to a persistently louder environment. Measuring sound by averaging it over a given time interval (Leq) provides a valuable metric for the soundscape at that location. However, it does not provide much information about the frequency or intensity of loud events within that time interval. The background noise was caused mostly by passenger vehicles, but was punctuated with much louder diesel semi-tractor trailers. Peak levels at some of the Highway sites reached over 100 dB, equivalent to an active construction site (OSHA 2013). Noise likely plays a role in pika stress, and our study only begins to probe this very complicated relationship. Further exploration of noise and pika

communication is instrumental to our understanding of pika stress and fitness along roads, and it could help inform future mitigation efforts.

Predators are a well-known source of stress in lagomorphs (Boonstra and others 1998, Yin and others 2017). High predation pressure increases stress hormones and changes pika behavior (Yin and others 2017). Lower hormone levels might indicate that those pikas are exposed to fewer predators in that habitat. However, the low stress levels from Highway sites are not likely due to an absence of predation pressure in the Highway habitat. While we did not quantify the difference in predation pressure between Highway and Rail Trail habitats, we confirmed the presence of several predator species (weasels, bobcat, domestic cat) at Highway sites (Figure 8). Our incidental observations of weasels (*Mustela frenata* and *M. erminea*) at multiple sites along the Highway during this study and in previous years suggests that pikas' primary predator frequents this roadside habitat.

Conclusion

Are highways stressful for pikas? We found that stress varied with habitat type, and conclude that Highway pikas were chronically stressed physiologically when compared to Talus or Rail Trail pikas. Among the potential stressors measured, we did not find a significant relationship between elevation or subsurface temperature on stress levels, but higher stress of Highway pikas than Talus is consistent with lower

elevation and higher temperatures. A significant relationship between longitude and fGCM suggests that higher stress levels occur in sites that typically experience drier climates. Noise was related to stress with sound measurements highest at Highway sites. We did not find definitive evidence of predator effects on stress levels, but camera captures of several predator species at Highway sites indicate that this habitat likely does not experience lower predation pressure than Rail Trail sites.

We recommend that future research expand the list of potential stressors measured. Diet, food availability, predator density, and pika density can prompt stress responses, and they would likely be different in roadside habitats than in natural habitats. We also suggest a greater sampling effort to illuminate any role that subsurface or surface temperatures may play in the pika stress response. Future work should make direct measurements of pika fitness in different habitat types. Reproduction and survival rates may be directly related to GC concentrations, or they could be indirectly affected by stressors; for example, stressed pikas may spend more time alert above the surface and less time caring for their offspring (Cyr and Romero 2007). Assessing behavior and the physical condition of pikas, while more invasive than the present study, would be helpful to understand how they respond to altered GC concentrations.

In our field study, we were unable to definitively identify the cause of the lower fGCM concentrations in the Highway habitat. However, evidence suggests that the mechanism responsible was a physiological downregulation of the HPA axis

responsiveness, not habituation. Distinguishing between these mechanisms is important. Habituation reduces the response only to a specific chronic stimulus (Rich and Romero 2005). Once a new stressor is introduced, the HPA axis reacts with an appropriate hormonal response (Dallman and Bhatnagar 2001). HPA downregulation changes an animal's stress response to any stimuli, not just the stressor to which it was chronically exposed (Walker and others 2006; Schöpper and others 2012). Under downregulation, the animal may not produce the necessary life-saving stress response to new stimuli, jeopardizing their survival. Incorrectly identifying these mechanisms could cause ecologists to overlook environmental stressors, and that could precipitate harmful policy decisions that fail to mitigate the impacts of human disturbance.

Knowing whether or not animals are experiencing stress and which environmental variables cause that stress will inform the design of WCSs to reduce those stressors, and therefore increase effectiveness in restoring connectivity. For example, our study suggests that pikas living along the highway are experiencing higher chronic stress levels. Their stress might be reduced (and fitness increased) by taking measures to reduce highway noise in and near WCSs. Similar recommendations might be worth considering for other low-mobility vertebrates.

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Appendix A – American Pika fecal collection dates and locations

Table 1. American Pika fecal pellet collection sites. Samples were collected from rocky sites in 3 habitats: Interstate 90 (Highway), the Palouse-to-Cascades Trail (Rail Trail), and natural talus (Talus).

Habitat	Collection Dates	Site Name	Site ID	Elevation (m)	# Pikas Sampled
Highway (n = 20)	July 27-August 15	Gold Creek Bridge	GCB03	769	2
		Keechelus Lake	KL01-03	759	4
		Mile Post 01	MP01	771	3
		Price Noble 07	PN07	770	1
		Price Noble 09	PN09	758	3
		Resort Creek	RC02	777	4
		Wolf Creek	WC	774	3
Rail Trail (n = 21)	July 31-August 1	Hyak 06	HY06	777	2
		John Wayne Trail 01	JWT01	770	2
		John Wayne Trail 02	JWT02	774	2
		John Wayne Trail 04	JWT04	773	2
		John Wayne Trail 05	JWT05	784	3
		John Wayne Trail 06	JWT06	776	2
		John Wayne Trail 07	JWT07	773	4
		John Wayne Trail 24	JWT24	781	3
		John Wayne Trail 26	JWT26	772	1
Talus (n = 22)	July 19-July 21	Mt. Amabilis 05	AM05	1143	2
		Mt. Amabilis 09	AM09	1186	1
		Mt. Amabilis 13	AM13	1100	1
		Mt. Amabilis 15	AM15	1047	1
		Gold Creek Trail 01	GCT01	837	4
		Gold Creek Trail 02	GCT02	834	2
		Hudson 01	HU01	707	3
		Hyak 02	HY02	879	3
		Hyak 04	HY04	799	3
		Rocky Run 01	RR01	1080	2

Appendix B – Species identified from camera traps positioned on pika haypiles

Table 2. Species identified from camera traps at sites along Interstate 90 (Highway) and the Palouse-to-Cascades Trail (Rail Trail). A camera was positioned roughly 5 m from a pika haypile at 7 Highway sites and 8 Rail Trail sites. Due to the size of one Highway site, we installed 2 cameras for adequate coverage of the area.

Species		Highway (n = 8)	Rail Trail (n = 8)
Lynx rufus	Bobcat	x	
Felis catus	House cat	x	
Canis lupus familiaris	Domestic Dog		x
Mustela frenata	Long-tailed Weasel	x	
Peromyscus keeni and/or *P. maniculatus*	Deer Mouse	x	
Neotoma cinerea	Bushy-tailed Woodrat		x
Callospermophilus saturatus	Cascade Golden-mantled Ground Squirrel	x	x
Otospermophilus beecheyi	California Ground Squirrel	x	
Tamiasciurus douglasii	Douglas Squirrel	x	
Neotamias amoenus and/or *N. townsendii*	Chipmunk	x	x
Marmota caligata	Hoary Marmot	x	
Lepus americanus	Snowshoe Hare		x
Cervus elaphus	Elk		x
Odocoileus hemionus	Mule Deer		x
Junco hyemalis	Dark-eyed Junco	x	
Turdus migratorius	American Robin		x